ATTACHMENT A

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IRES-Dependent Second Gene Expression Is Significantly Lower Than Cap-Dependent First Gene Expression in a Bicistronic Vector

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The internal ribosome entry site (IRES) has been widely used to coexpress heterologous gene products by a message from a single promoter. However, little is known about the efficiency of IRES-dependent second gene expression in comparison with that of first gene expression. This study was undertaken to characterize the relative expression of IRES-dependent second gene in a bicistronic vector, which was derived from the 5′ untranslated regions of the encephalomyocarditis virus (EMCV). IRES-dependent second gene expression was compared with cap-dependent first gene expression in several cultured cell lines and in mouse liver *in vivo*. The expression of the IRES-dependent second gene ranged from 6 to 100% (in most cases between 20 and 50%) that of the first gene. Second gene expression in a plasmid without the IRES was 0.1–0.8% (with some exceptions) that of the first gene. These findings have important implications for the use of IRES, i.e., care should be taken regarding the decreased capacity of IRES-dependent downstream gene expression as well as in determining which gene should be positioned as the first or second gene in a bicistronic vector.

Key Words: **IRES; EMCV; bicistronic vector; gene therapy.**

INTRODUCTION

Internal ribosome entry site (IRES) sequences allow the initiation of translation in a cap-independent manner: ribosomes bind internally at the initiating AUG without scanning the 5′ nontranslated region of the transcript (1). The function of IRES was first shown in the 5′ noncoding region of poliovirus RNA (2) and has been demonstrated in picornavirus [encephalomyocarditis virus (EMCV)] RNAs and other viral messages (3–6). In addition, some cellular mRNAs have been shown to possess IRES function (7). IRES derived from the 5′ nontranslated regions of the EMCV genome are the most widely used in gene therapy and gene transfer experiments (1). EMCV IRES have a higher translation efficiency than other IRES sequences, including those from hepatitis A and C viruses, poliovirus, human rhinovirus, and foot-and-mouth disease virus (6). Furthermore, EMCV IRES are functional in a variety of cultured cell types, unlike those of poliovirus and rhinovirus (5).

There are currently two methods to coexpress heterologous gene products in a single vector, i.e., using either independent promoters or IRES sequences (1), although heterologous gene products can be expressed by alternative splicing or reinitiation of translation in a limited number of cases. Promoter interference sometimes occurs with the use of heterologous promoters, i.e., transcription from one promoter suppresses transcription from another (8–10). The IRES method eliminates such problems, as more than two genes connected by IRES sequences can be efficiently expressed from a single promoter (3, 4, 11).

EMCV IRES is currently in wide use, especially in plasmid, retrovirus, and adeno-associated virus vectors (12–20). However, little is known about the efficiency of IRES-dependent second gene expression relative to first gene expression. In this study, we analyzed the respective efficiencies of expression of a cap-dependent first gene and an IRES-dependent second gene in a bicistronic vector in which each gene could be translated with maximal efficiency. Three model genes, luciferase, secreted alkaline phosphatase (SEAP), and chloramphenicol acetyltransferase (CAT), were used to compare expression efficiency.

MATERIALS AND METHODS

Plasmid. We used the bicistronic plasmids pL-IRES-SEAP1, pSEAP-IRES-L1, pL-IRES-CAT1, and pCAT-IRES-L1, which contain the cytomegalovirus enhancer/chicken β-actin (CA) promoter (kindly provided by Dr. J.

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Miyazaki, Osaka University, Osaka, Japan) (21); the reporter gene (luciferase, SEAP, or CAT); IRES; and SV40 late poly(A) signal (Fig. 1). The IRES sequence was derived from pT7-IRES-L(+)1, itself a derivative of pT7-IRES-L (22), which contains a complete sequence identical to pT7EMCAT (23) (kindly provided by Dr. B. Moss, National Institutes of Health, Bethesda, MD). The luciferase, SEAP, and CAT genes were derived from pGL3-Control (Promega, Madison, WI), pSEAP2-Control (Clontech, Palo Alto, CA), and pT7EMCAT (23), respectively. The start (ATG) codons of all first and second genes were adjusted to mirror those of β-actin and EMCV, respectively. The plasmids pL-SEAP and pSEAP-L do not contain IRES but rather have Kozak consensus sequences surrounding the start codon of the second gene (Fig. 1) (24, 25). The SEAP gene and luciferase gene in pSEAP-L2 have a junction sequence that is identical to the sequence between the luciferase gene and SEAP gene in pL-SEAP.

Cells. HeLa (human epitheloid carcinoma, cervix), L (Lcl1D, mouse fibroblast), and CHO (Chinese hamster ovary) cells were cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), minimum essential medium (MEM) supplemented with 10% FCS, and MEM alpha medium supplemented with 10% FCS, respectively.

Gene transfer into cultured cells. Cells (5×10^4: HeLa, L, and CHO) were seeded into a 24-well dish. On the following day, each vector was transfected with SuperFect (Qiagen) according to the manufacturer's instructions. After 2 h the cells were washed and cultured with fresh medium every 24 h. Forty-eight hours later, luciferase and CAT activity in the cells and SEAP activity in the medium were determined. All activities were corrected by transfection of the control plasmid pUC18. All transfection experiments were repeated at least three times with similar results.

Gene transfer into mouse liver. In vivo transfection of mouse liver was performed according to the method of Liu *et al.* (26). The mice (male Balb/c, 6 weeks old) were injected via the tail vein with 10 μg of pL-IRES-SEAP1, pSEAP-IRES-L1, pL-IRES-CAT1, or pCAT-IRES-L1 in 2.0 ml of saline for 5 s. Liver and blood samples were recovered 48 h postinjection, and luciferase and CAT activity in the liver and SEAP activity in the serum were determined. All activities were corrected by transfection of the control plasmid pUC18. Gene expressions in spleen, heart, and kidney were 0.008, 0.024, and 0.029%, respectively, that measured in liver.

Reporter gene assay. Luciferase, SEAP, and CAT activities were measured using a luciferase assay system (PicaGene LT2.0, Toyo Inki Co. Ltd., Tokyo, Japan), a Great EscAPe SEAP chemiluminescence detection kit (Clontech), and a CAT ELISA kit (Boehringer Mannheim, Tokyo, Japan), respectively. Protein content was measured with a Bio-Rad assay kit using bovine serum albumin as standard.

RESULTS

Structures of the First and Second Gene Fragments

To compare the IRES-dependent second gene expression with that of cap-dependent first gene expression, we constructed several vectors having optimal translation efficiencies for first and second gene expression, respectively (if the first and second genes have a nonoptimal translation start codon, an accurate comparison cannot be done). The adjustment of the ATG start codon of the gene of interest (first gene) to mirror that of β-actin is believed to produce maximal translation efficiency in vectors containing the CA promoter. For example, we have previously reported that deletion of the 5′ untranslated region of luciferase and human tumor necrosis factor α increases the expression of these genes 20- and 5- fold, respectively (27, 28).

We used EMCV IRES derived from pT7EMCAT (pTM1) developed by Moss *et al.* (23, 29) for its convenience in cloning. This IRES fragment has an engineered *Nco*I site placed at the EMCV initiation codon, but it has the same translation efficiency as that found in wild-type EMCV (11). The relative levels of IRES-driven gene expression were higher when the coding sequence of the gene of interest was placed directly at the 3′ end of EMCV IRES than when the gene and the IRES were separated by a 5′ untranslated fragment of the gene (11). Therefore, all first and second gene coding sequences of the plasmids in this study were placed immediately downstream of the start codons of β-actin and EMCV, respectively.

Second gene expression in a plasmid without the IRES sequence (pL-SEAP, pSEAP-L) (Fig. 1) was also examined. In these plasmids, the start codon of the second gene was surrounded by a Kozak consensus sequence (24, 25) to achieve the highest levels of gene expression, which come from either the reinitiation of the ribosomal complex for translation (30) or a spliced message (11, 31).

Comparison of First and Second Gene Expression with IRES in Vitro and in Vivo

Expression of the cap-dependent first gene was compared with that of the IRES-dependent second gene in HeLa, L, and CHO cells transfected by pL-IRES-SEAP1 or pSEAP-IRES-L1 (Fig. 2). Luciferase activity in all cell lines transfected by pSEAP-IRES-L1 was approximately 3.5–7 times lower than that in cells transfected by pL-IRES-SEAP1. Similarly, SEAP activity in all cell lines transfected by pL-IRES-SEAP1 was approximately 3–6 times lower than that in cells transfected by pSEAP-IRES-L1.

To determine whether the phenomenon illustrated in Fig. 2 can be generalized to other genes in this arrangement, first and second gene expressions were compared using another bicistronic plasmid with luciferase and CAT genes (pL-IRES-CAT1, pCAT-IRES-L1) (Fig. 3). Luciferase activity in HeLa or CHO cells transfected by pCAT-IRES-L1 was about 7 or 2 times lower than that in cells transfected by pL-IRES-CAT1, respectively (Figs. 3A-1 and 3A-3). In L cells, however, pCAT-IRES-L1 expressed luciferase as efficiently as pL-IRES-CAT1 (Fig. 3A-2). In contrast, CAT expression in all cell lines transfected by pL-IRES-CAT1 was lower than that in cells transfected by pCAT-IRES-L1 (Fig. 3B), particularly in L or CHO cells transfected (approximately 8 or 16 times lower, respectively).

Next, to determine how cap-dependent translation compares with IRES-dependent translation in a clinically relevant model, we used a hydrodynamics-based method of expressing transgenes in the liver of mice by tail vein injection of plasmid DNA (26). In experiment 1 (Figs. 4A and 4B), luciferase and SEAP activities after injection of pL-IRES-SEAP1 or pSEAP-IRES-L1 were examined. Second gene expression was approximately 3–6 times lower than first gene expression in both cases. In experiment 2 (Figs. 4C and 4D), we assayed luciferase and CAT activities after injection of pL-IRES-CAT1 or pCAT-IRES-L1. In this experiment, second gene expression was approximately 17 or 1.7 times lower than first gene expression. Thus, the *in*

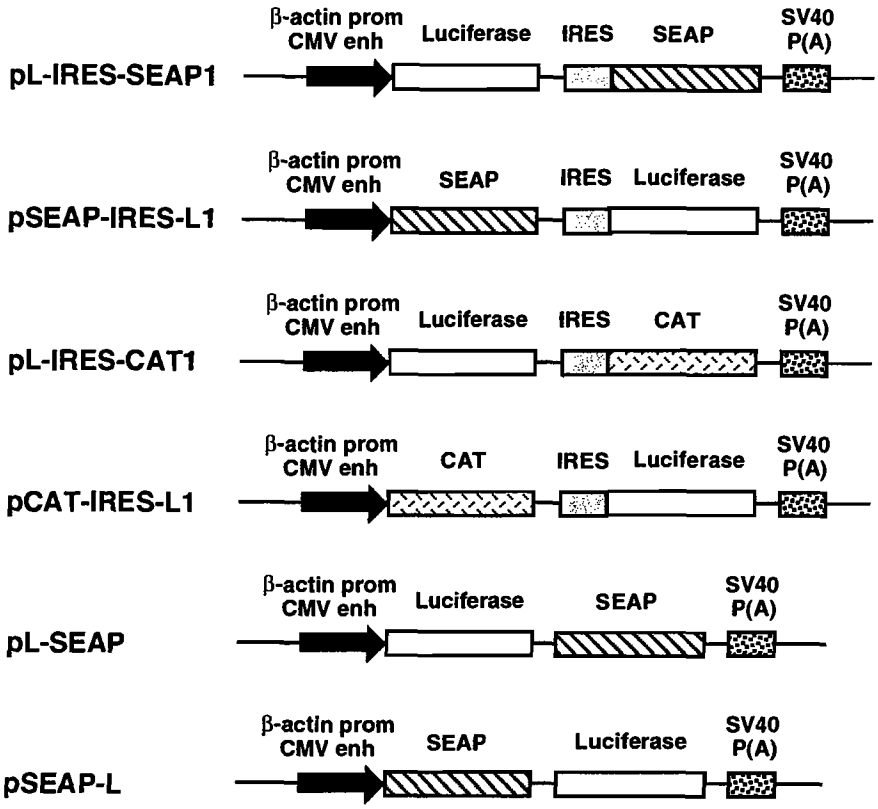

FIG. 1. Plasmid structure. To obtain maximal expression of both the first and second genes in the vector with IRES, the start codon of the first or second gene was adjusted to that of β-actin or EMCV, respectively. In the IRES(−) vector, the second gene contains a Kozak consensus translation initiation site.

vivo results correlated well with those obtained from the *in vitro* experiments.

Taken together, these results suggest that IRES-dependent second gene expression is less efficient than cap-dependent first gene expression and that expression levels of the second gene can range from 6 to 100% (generally 20–50%) those of the first gene.

Comparison of First and Second Gene Expression without IRES

To examine possible effects of IRES on second gene expression, we next measured second gene expression by the plasmid without IRES (Fig. 1, Table 1). In these vectors, expression of the second gene can be expected to occur with ribosome scanning, which targets the start codon of the second gene rather than that of the first gene (30) and/or the spliced mRNA (11, 31).

With respect to expression of the first gene, no remarkable differences were observed between plasmids with IRES and plasmids without IRES; i.e., pL-IRES-SEAP1 vs pL-SEAP or pSEAP-IRES-L vs pSEAP-L (Fig. 2, Table 1). In contrast, luciferase activity in all cell lines transfected by pSEAP-L was over 100 times lower than that in cells transfected by pL-SEAP. SEAP expression in L and CHO cells transfected by pL-SEAP was also over 100 times less than that in cells transfected by pSEAP-L. These results suggest that in the vector without IRES, expression of the second gene is much less efficient than that of the first gene or that of the IRES-dependent second gene. SEAP expression in HeLa cells, however, showed an exceptional pattern. HeLa cells transfected by pL-SEAP expressed more SEAP than did cells from the same line that were transfected by pL-IRES-SEAP1 (Fig. 2B-1, Table 1) and half as much SEAP as cells from the same line transfected by pSEAP-L. In this context, the second gene was expressed efficiently.

It has recently been reported that a short (36 bp) synthetic intercistron can reinitiate translation and efficiently drive second gene expression (32). We speculated that a short junction sequence [TAA(stop)TTCTAGCCTCGAGGAATTCGCCCACCATG(start)] between the luciferase (first) and SEAP (second) genes in pL-SEAP could play such a role in HeLa cells, which would explain why high SEAP activity was observed in HeLa cells transfected by pL-SEAP. To investigate this possibility, pSEAP-L2, which has exactly the same junction sequence as pL-SEAP, was constructed, and the expression of SEAP and luciferase in HeLa cells transfected by pSEAP-L2 was examined (Fig. 5). However, higher levels of luciferase (second gene) expression were not observed, suggesting that the junction sequence between the luciferase and SEAP genes in pL-SEAP did not enhance the reinitiation of translation.

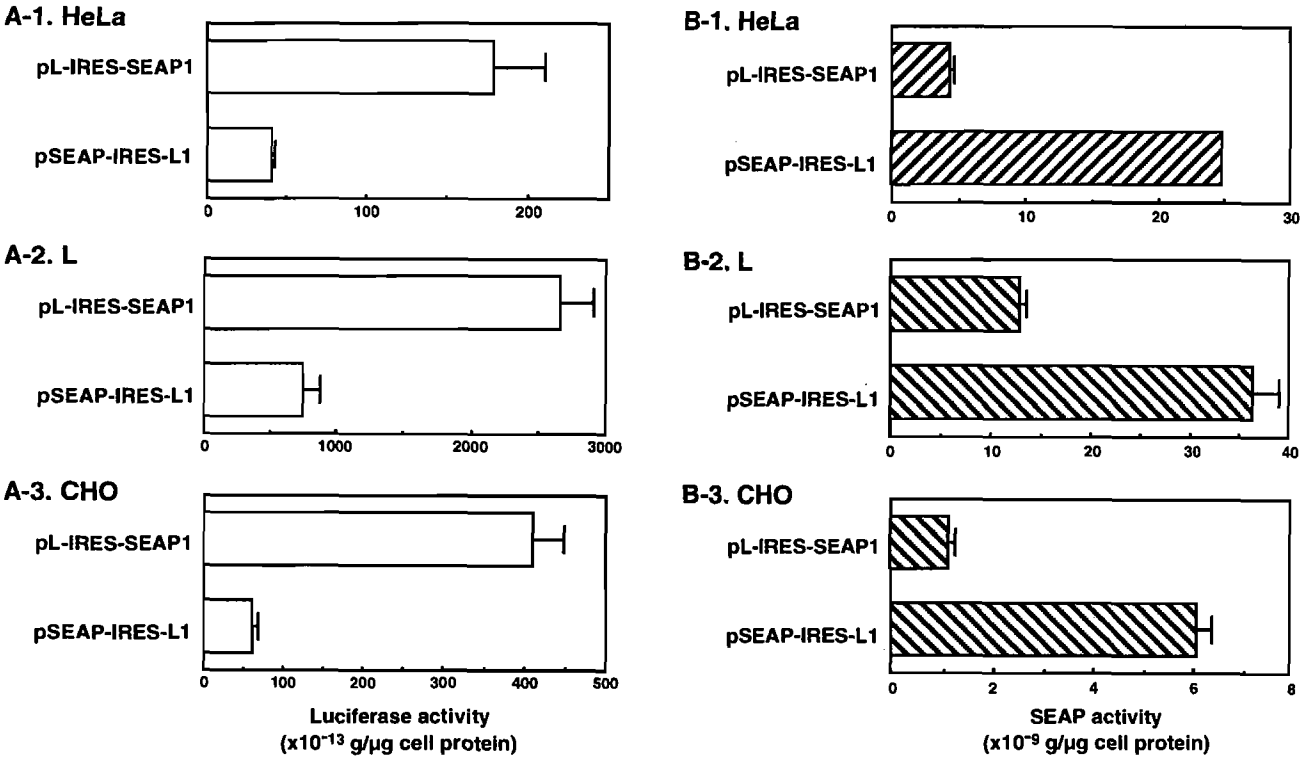

FIG. 2. *Comparison of luciferase and SEAP expression in the cells transfected with pL-IRES-SEAP1 or pSEAP-IRES-L1.* pL-IRES-SEAP1 or pSEAP-IRES-L1 was transfected into HeLa (A-1, B-1), L (A-2, B-2), or CHO cells (A-3, B-3). After 48 h in culture, luciferase (A-1, A-2, A-3) activity in the cells and SEAP (B-1, B-2, B-3) activity in the medium were determined. All data represent means ± SD of three experiments.

DISCUSSION

Second Gene Expression with IRES

The aims of this study were to identify the relative capacities of EMCV IRES to mediate the expression of the downstream gene in the bicistronic vector in comparison with the expression of the first gene, which is translated in a cap-dependent manner. To our knowledge, this is the first report which examined the quantitative efficiency of IRES-dependent second gene expression, although IRES is now widely used to express multiple proteins from a single transcriptional unit (12–20). Expression of the IRES-dependent second gene was less efficient than that of the first gene under both *in vitro* (HeLa, L, and CHO cells) and *in vivo* (mouse liver) conditions, with the expression level varying from 6 to 100% (relative to first gene expression), depending on cell types and reporter genes (Table 2).

In many gene transfer experiments, the cDNA of the gene of interest is simply inserted after the promoter or IRES sequence. In such cases, the translation efficiency of the gene of interest is not optimized (27, 28). Therefore, the way in which the plasmid is constructed can modulate the expression of the IRES-dependent second gene relative to that of the first gene. Both the capacity of IRES and the translation efficiency should be taken into consideration in relation to the relative expression of the first and second genes. If the same levels of expression are desired of the first and second genes, decreased efficiency of the translation of the first gene by non-Kozak sequence or a change in the ATG start codon (33) would be possible strategies. For example, the same level of expression of the first and second genes is required for interleukin-12 (IL-12) (34). The expression vector used for IL-12 employs IRES to express both p35 and p40. However, excess expression of p40 can form a homodimer, which then prevents the efficient expression of IL-12. IRES is also widely used to express a drug-selective gene (e.g., antibiotics such as neomycin) in the vector, the structure of which is [promoter]–[gene A]–[IRES]–[selective gene] (15). Our results suggest that, in this type of vector, a lower concentration of drug should be used than that used with a vector in which the drug-selective gene is translated in a cap-dependent manner. In previous research studies, a cytokine gene and a suicide gene have been expressed from a single vector using IRES to provide effective cancer gene therapy (14, 17, 19, 20). In some studies, the cytokine gene and suicide gene are positioned as the first gene and second gene, respectively (17, 19, 20). However, in another study, the suicide gene is positioned as the first gene and the cytokine gene is positioned as the second gene (14). Other information from our study suggests that in these types of cases, the gene with higher anticipated expression should be positioned as the first gene.

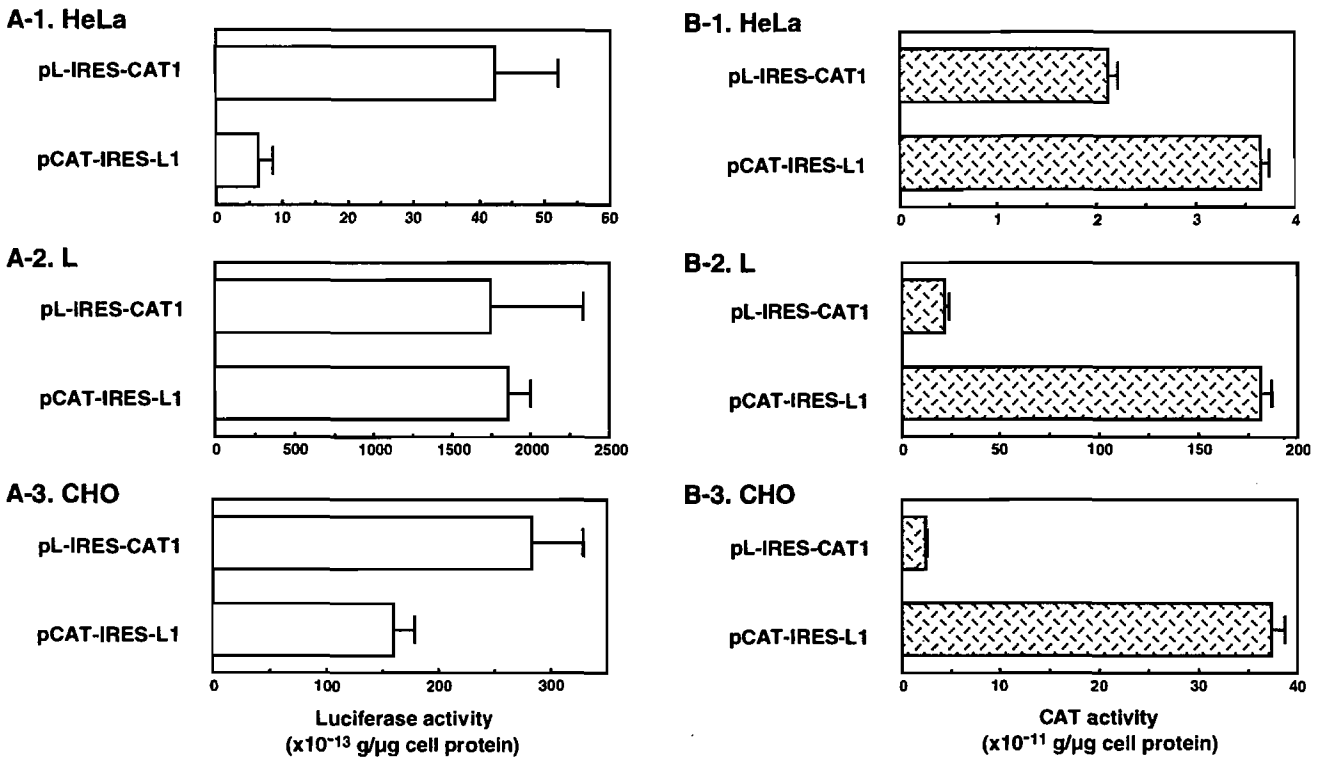

FIG. 3. Comparison of luciferase and CAT expression in the cells transfected with pL-IRES-CAT1 or pCAT-IRES-L1. pL-IRES-CAT1 or pCAT-IRES-L1 was transfected into HeLa (A-1, B-1), L (A-2, B-2), or CHO cells (A-3, B-3). After 48 h in culture, luciferase (A-1, A-2, A-3) and CAT (B-1, B-2, B-3) activities in the cells were determined. All data represent means ± SD of three experiments.

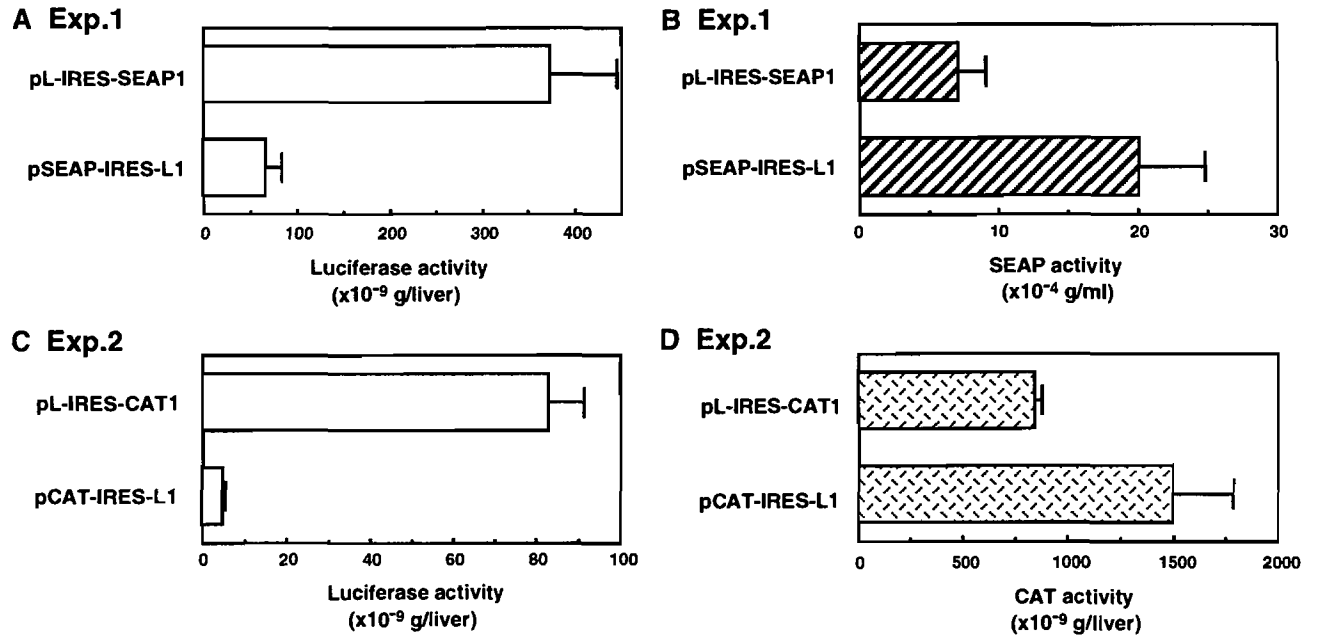

FIG. 4. Comparison of first and second gene expression in mice liver *in vivo*. Mice were injected with 2.0 ml of saline containing 10 μg of pL-IRES-SEAP1 or pSEAP-IRES-L1 (Exp. 1: A, B) or 10 μg of pL-IRES-CAT1 or pCAT-IRES-L1 (Exp. 2: C, D). Animals were killed 48 h after injection and luciferase and CAT activities in the liver and SEAP activity in the serum were determined. All data represent means ± SE of five experiments.

TABLE 1

Comparison of Luciferase and SEAP Expression in Cells Transfected with pL-SEAP or pSEAP-L

Cell	Plasmid	Luciferase activity ($\times 10^{-13}$ g/μg cell protein)	SEAP activity ($\times 10^{-9}$ g/μg cell protein)
HeLa	pL-SEAP	138.6 ± 23.6	12.37 ± 0.46
	pSEAP-L	1.1 ± 0.3	25.48 ± 0.80
L	pL-SEAP	2270 ± 156	1.22 ± 0.04
	pSEAP-L	5.8 ± 0.5	50.66 ± 3.39
CHO	pL-SEAP	231.3 ± 13.4	0.17 ± 0.01
	pSEAP-L	0.3	5.04 ± 0.36

Note. pL-SEAP or pSEAP-L was transfected into HeLa, L, or CHO cells. After 48 h in culture, luciferase activity in the cells and SEAP activity in the medium were determined. All data represent means ± SD of three experiments.

Second Gene Expression without IRES

The relative efficiency of expression of the second gene in the vector without IRES was less than 1% that of expression of the first gene, and the presence of IRES increased second gene expression by more than 10- to 100-fold (Fig. 2, Tables 1 and 2). The start codon of the second gene in this study had a Kozak consensus sequence (24). If the sequence around the start codon had been less efficient for translation, the expression may have decreased more. This information could be useful in cases in which only minimal expression of a foreign gene is desirable.

Unexpectedly, SEAP (second gene) activity in HeLa cells transfected by pL-SEAP was higher than the IRES-dependent SEAP activity in HeLa cells transfected by pL-IRES-SEAP1 (Fig. 2, Table 1). We speculated that a short junction sequence between the luciferase (first) and SEAP (second) genes in pL-SEAP might reinitiate translation of SEAP, similar to the observations described by Havenga *et al.* (32). However, a junction sequence in our system did not have the ability to enhance the reinitiation of translation (Fig. 5). Another possibility is that the spliced mRNA caused the efficient second gene (SEAP) expression seen in HeLa cells, although this is unlikely because first gene (luciferase) expression in HeLa cells was similar between pL-IRES-SEAP1 and pL-SEAP (Fig. 2, Table 1). Therefore, depending on the gene and cell combination used, it is likely that reinitiation of second gene translation (24) could be efficient, although this might be rare. The precise mechanism leading to the observed higher second gene expression in HeLa cells is unknown.

In summary, we have demonstrated that in a bicistronic construct with IRES, the capacity of IRES-dependent second gene expression is usually significantly lower than that of cap-dependent first gene expression. This information must be taken into account for the use of IRES in gene transfer and gene therapy experiments.

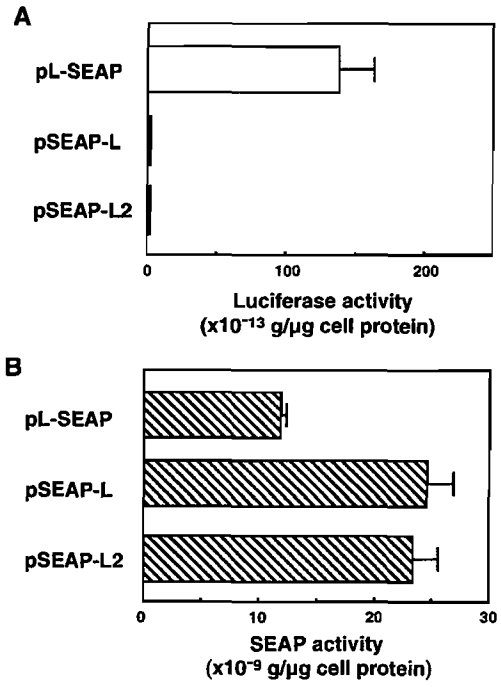

FIG. 5. Comparison of luciferase and SEAP expression in HeLa cells transfected with pL-SEAP, pSEAP-L, or pSEAP-L2. pL-SEAP, pSEAP-L, or pSEAP-L2 was transfected into HeLa cells. After 48 h in culture, luciferase (A) and SEAP (B) activities in the cells were determined. All data represent means ± SD of three experiments.

TABLE 2

Relative Second Gene Expression Level

Gene	Activity (%)
1st gene	100
IRES(+)-2nd gene	6.4–100
IRES(−)-2nd gene	0.1–0.8[a]

Note. The relative efficiency of second gene expression is expressed as percentage of first gene expression. In the IRES(+)-second gene, second gene expression was compared with the first gene expression by the vector with IRES. In the IRES(−)-second gene, second gene expression was compared with the first gene expression by the vector without IRES.

[a] Exceptionally, in HeLa cells, second gene (SEAP) expression by pL-SEAP showed 48% of first gene (SEAP) expression by pSEAP-L.

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